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# Decreased protein and puromycinyl-peptide degradation in livers of senescent mice

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Liver protein-degradation rates were determined in young and old C57B1 mice by the method of Swick & Ip [(1974) *J. Biol. Chem.* **249**, 6836–6841]. The results indicated a marked age-related increase in the half-lives of short-lived proteins in the nuclear, mitochondrial, lysosomal and 100000*g*-supernatant cellular fractions and in total trichloroacetic acid-precipitable proteins. The efficiency of the degradation system in removing aberrant proteins from livers of young and old mice was tested. The time required for 50% disappearance of puromycinyl-peptides changed from about 20 min in 6-month-old mice to approx. 150 min in 24-month-old animals. These findings suggest that in old animals the proteolytic activity involved in degradation of aberrant proteins, and presumably of 'native' proteins, is markedly defective.

Accumulation of faulty enzyme molecules has been reported for various enzymes in a number of aging systems (Gershon, 1979). The accumulation of these aberrant molecules, which are antigenically reactive but catalytically inactive, could be due to a loss of efficiency of the degradation system in old animals (Gershon *et al.*, 1979). Indeed, Reznick & Gershon (1979) and Prasanna & Lane (1979) have demonstrated a 4–5-fold increase in protein half-lives in old as compared with young nematodes. Increases in the half-lives of enolase and aldolase were also reported in the aged nematodes (Sharma *et al.*, 1979; Zeelon *et al.*, 1973). We have shown a 50% increase in the half-life of aldolase in old mice (Reznick *et al.*, 1981).

Amils *et al.* (1977) showed that abnormal proteins such as puromycinyl-peptides were degraded much more slowly in rapidly growing livers of animals recovering from protein deprivation than in the protein-deprived controls. This slow-down in degradation of abnormal proteins was directly related to the large decrease in the overall rate of total liver protein degradation during liver growth (Scornik & Botbol, 1976; Conde & Scornik, 1977).

The present investigation was undertaken in order to examine, by studying the rate of degradation of total proteins, the possibility that the protein-degradation system could also be impaired in aging mammals. Furthermore, it was intended to study the rate of disposal of puromycinyl-peptides in livers of old animals as a measure of the physiological state of the protein-degradation system in aging organisms. Disposal of abnormal proteins is considered to be one of the main physiological functions of the protein-degradation system (cf. Goldberg & St. John, 1976).

## Materials and methods

### *Animals*

C57B1/6J female mice which were raised and housed under specific pathogen-free conditions were used throughout the experiment. The average weights of representative groups of 18 young animals (6 months) and 17 old animals (24–25 months) were $24.3 \pm 1.8$ and $27.8 \pm 2.1$ g, respectively.

### *Preparation of homogenates and subcellular fractions*

Individual livers were washed in ice-cold iso-osmotic solution (0.25 M-sucrose). Homogenization was carried out in 3 vol. of the same solution. Five subcellular fractions were prepared by differential centrifugation by the procedure of Novikoff & Heus (1963). All the fractions were washed three times and resuspended in 3 ml of iso-osmotic medium, except for the microsomal fraction, which was resuspended in 6 ml of the medium. Samples (100, 150 and 200 $\mu$l) were processed for radioactive determination as described below. Total precipitable proteins were extracted with hot trichloroacetic acid and organic solvents as described by Scornik (1974).



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*Determination of incorporation of labelled compounds*

$NaH^{14}CO_3$ (40Ci/mol) and [$^3$H]puromycin (5.71 Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

$NaH^{14}CO_3$ (100 or 200$\mu$Ci per animal) was administered intraperitoneally. In each experiment at the indicated times after injection, three or four animals of each age group were killed, their livers removed, homogenized and fractionated. All protein preparations were suspended in 10% (w/v) trichloroacetic acid and washed extensively with 5% trichloroacetic acid on Whatman GF/A filters. The filters were then dried, placed in 10ml of scintillation liquid (Swick & Ip, 1974; Scornik, 1974) and counted for radioactivity in a Packard Tri-Carb spectrometer.

[$^3$H]Puromycin was injected intraperitoneally, at a dose of 20$\mu$Ci per animal in 0.2ml of 0.9% NaCl. Animals in both age groups received the same dose, as their weight did not vary significantly. At various time intervals mice were killed, livers removed within 10–15s and immediately frozen in liquid air to prevent both further incorporation of label and degradation of peptides. Subsequently, livers were weighed and homogenized with a Teflon/glass homogenizer in 0.05M-phosphate buffer (pH 7.5) containing 0.25M-sucrose (1:5, w/v). The homogenates were spun at 100000*g* for 70min; 0.2ml samples of the supernatant were adjusted to a final concentration of 10% trichloroacetic acid at 4°C. The pellets obtained by centrifugation at 3000*g* for 10min were washed with 3 × 2ml of 10% trichloroacetic acid. The final precipitates were dissolved in 2ml of 0.5M-NaOH and samples were counted for radioactivity in Triton/toluene scintillation liquid (Reznick & Gershon, 1979).

## Results

*Degradation rates of proteins in livers of mice of various ages*

$NaH^{14}CO_3$ (100 or 200$\mu$Ci per animal) was administered to groups of 4-, 18- and 28-month-old mice. In each individual experiment, 4h after injection three or four animals of each age group were killed and radioactivity in the various cellular fractions of their livers was determined as described in the Materials and methods section. In each experiment the same procedure was followed on days 1, 3 and 5. The results were plotted semilogarithmically (Fig. 1) and the times required for disappearance of 50% of the radioactivity were determined. The results obtained in the 18-month-old group were essentially the same as those of the 4-month-old animals. Thus only the results of the 4- and 28-month-old animals are depicted in Fig. 1 and Table 1. The results are the average of three individual experiments.

Except for the proteins of the microsomal fraction, there was a significant slow-down in the rate of protein degradation in the first 3 days in all the subcellular fractions of livers of old animals and in total trichloroacetic acid-precipitable proteins. However, enhanced degradation of proteins occurred in old animals between days 3 and 5. This unusual and interesting phenomenon is discussed below.

Consequently, it was decided to study whether this observed retardation of protein degradation in the first 3 days was related to an irreversible loss of the functional capacity of the degradation system or rather was due to a controlled slow-down in protein turnover in old animals. This question was approached by testing the efficiency of removal of puromycinyl-peptides in livers or young and old animals.

*Rate of removal of puromycinyl-peptides*

In all experiments young (5 months) and old (24 months) mice were injected with [$^3$H]puromycin. At the times indicated in the pertinent Figures, animals of each age group were killed and the radioactivity in their liver proteins was determined. Fig. 2 demonstrates the results of initial experiments which show that the rates of incorporation of labelled puromycin into liver proteins are linear for the first 12min and are similar in both young and old mice.

In order to determine the rate of disappearance of label as accurately as possible, incorporation and reutilization after the initial pulse period had to be minimized. Thus the effect of administration of unlabelled puromycin on the incorporation of [$^3$H]puromycin was studied. Initially, two concentrations (0.4 and 4.0mg per mouse) of unlabelled

Table 1. *Degradation of total proteins in various cellular fractions of livers of young and old C57B1 mice*

The values given are close approximations derived from Fig. 1, since the kinetics of disappearance of label did not always follow first-order kinetics. This is due to the heterogeneity of the half-lives of the various proteins which comprise each fraction (for discussion see Garlick *et al.*, 1976).

| | Time (days) required for disappearance of 50% of $^{14}$C label | | |
|---|---|---|---|
| Protein fraction | Young | Old | Increment (%) |
| Nuclear | 1.60 | 3.05 | 90.6 |
| Mitochondrial | 1.85 | 3.00 | 62.2 |
| Lysosomal | 1.00 | 2.10 | 110 |
| Microsomal | 1.25 | 1.60 | 28 |
| Total soluble | 1.90 | 3.10 | 63.2 |

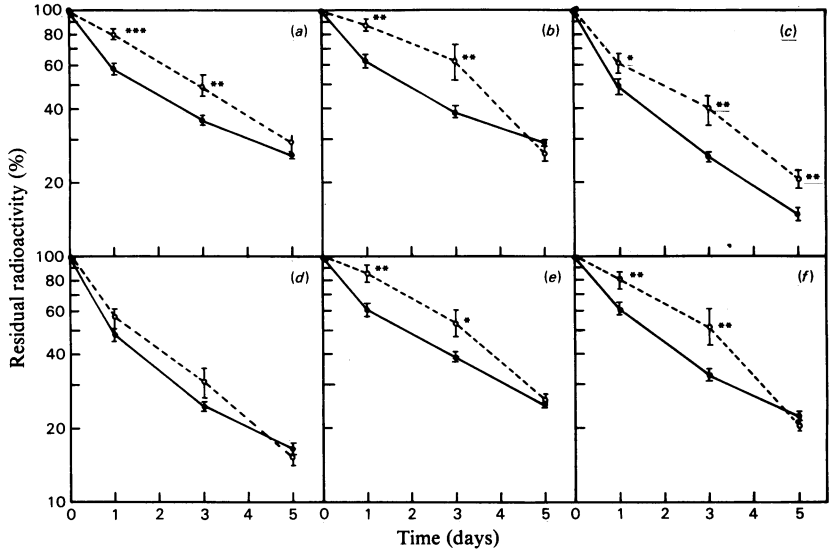


Fig. 1. *Decay of radioactivity in proteins of subcellular fractions and total trichloroacetic acid-precipitable proteins in livers of 3–5-month-old* (●) *and 27–29-month-old animals* (○)

Values represent the means ± S.E.M. for three pooled experiments. In the first, mice received 100 $\mu$Ci of $NaH^{14}CO_3$/20g body weight. In the second and third experiments, each mouse received 200 $\mu$Ci of $NaH^{14}CO_3$. In each experiment three or four young and two or three old animals were killed at the indicated times after injection. The results are expressed as percentages of the radioactivity values obtained in liver proteins 4h after the injection of $NaH^{14}CO_3$. Depending on the various cellular fractions, these values were between 776 and 3343c.p.m./mg of protein in livers of young and 980–3208c.p.m./mg of protein in livers of old animals. The differences in percentage of residual radioactivity between young and old groups at the different time points were tested for significance by Student's *t* test for independent groups. The following degrees of significance are denoted on the Figure: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Fractions: (*a*) nuclear; (*b*) mitochondrial; (*c*) lysosomal; (*d*) microsomal; (*e*) 100000*g* supernatant; (*f*) total acid-precipitable proteins.

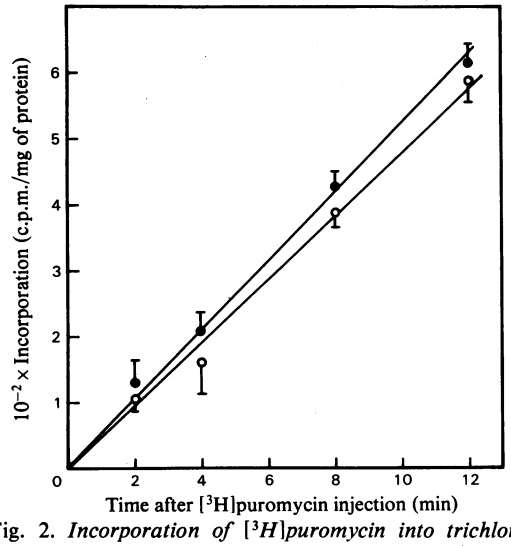


Fig. 2. *Incorporation of* [$^3$H]*puromycin into trichloroacetic acid-precipitable liver proteins in young* (●) *and old* (○) *animals*

Conditions for radioactivity incorporation and preparation of puromycinyl-peptides for scintillation counting are described in the Materials and methods section. Each point represents an average value obtained from two animals.

puromycin were tested for their suitability in chase experiments; 0.4mg had only slight effect, whereas 4mg caused an 80% inhibition of [$^3$H]puromycin incorporation within 4min. Inhibition of incorporation was studied by first injecting young and old animals with 4mg of unlabelled puromycin, and 4min later 20 $\mu$Ci of [$^3$H]puromycin was injected. After 4min more the amount of acid-precipitable label was measured; 100% incorporation was determined as the amount of acid-precipitable label in proteins 4min after the injection of 20 $\mu$Ci of [$^3$H]puromycin. It was thus found that already at 4min incorporation was inhibited by 75.4% and 81.1% in young and old animals respectively. This inhibition was maintained in both age groups at 8 and 12min after administration of labelled puromycin. It was thus shown that it was feasible to study the rate of degradation of puromycin-peptides in the livers of young and old animals.

In order to determine the rate of degradation, an injection of 4mg of unlabelled puromycin was given 12min after the administration of 20 $\mu$Ci of [$^3$H]-puromycin per mouse. At the indicated times, three animals were killed and the amount of label in soluble hepatic proteins was determined for each animal. A semi-logarithmic plot of the results (Fig.

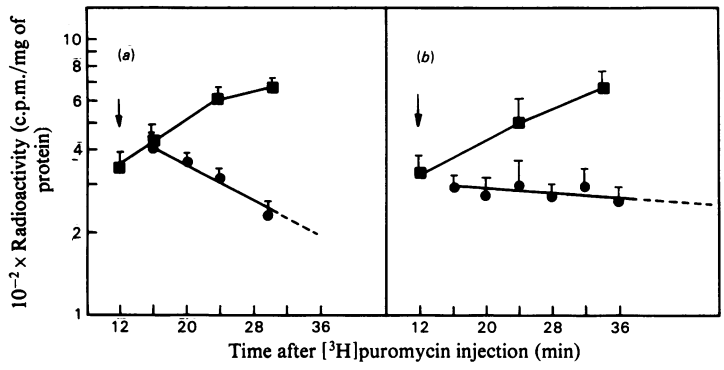


Fig. 3. *Degradation rates of [$^3$H]puromycinyl-peptides after administration of unlabelled puromycin in (a) young and (b) old animals*

At 12min after administration of [$^3$H]puromycin (20$\mu$Ci/0.2ml per animal), half of the animals were injected with 4mg of unlabelled puromycin in 0.2ml of saline (0.9% NaCl) and the other half with saline alone. Animals were killed at the indicated times and livers were removed within 15s and instantly frozen in liquid air. The amount of radioactive label present in trichloroacetic acid-precipitated proteins was determined by scintillation counting. Each point represents the average value from three different animals. The vertical bars represent S.D. values. ■, No unlabelled puromycin added; ●, unlabelled puromycin added.

3) indicates that the time required for 50% disappearance of labelled puromycinyl-peptides in young animals is approx. 20min. The time required for a similar loss of label in livers of old animals is vastly different and can be estimated by extrapolation to be of the order of 150min.

## Discussion

Several investigations have shown that abnormal proteins containing amino acid analogues or puromycinyl-peptides are degraded much more rapidly than normal peptides in bacteria (Goldberg, 1972), reticulocytes (McIlhinney & Hogan, 1974) and other systems (Knowles *et al.*, 1975; Knowles & Ballard, 1976). Amils *et al.* (1977) demonstrated that under conditions which caused a large decrease in the overall rates of total protein degradation in liver, the rate of scavenging of abnormal proteins such as puromycinyl-peptides was also considerably decreased. Decreased protein degradation has been shown to occur during liver regeneration (Scornik & Botbol, 1976) and under conditions of starvation and re-feeding (Conde & Scornik, 1977).

The present work shows for the first time that, in the liver of aging animals, concomitant with the decrease in the capacity to degrade 'native' proteins the disposal of aberrant peptides becomes severely defective. We have previously shown that in aging nematodes (Reznick & Gershon, 1979) proteins containing canavanine and fluorotryptophan were degraded at a much lower rate. In mouse liver the half-life of aldolase has been shown by us to be significantly increased (Reznick *et al.*, 1981).

The general defect described here apparently applies mainly to proteins with relatively short half-lives. It is obvious from our results that, in all cellular fractions of livers of old animals, proteins with half-lives of up to 3 days are degraded at considerably lower rates. However, in old animals between days 3 and 5 the proteins are degraded at an enhanced rate. The curves for young and old animals show substantial divergence on days 1 and 3, but converge on day 5 (Fig. 1). Between days 5 and 8 the two curves become parallel (Lavie, 1978). The enhancement in degradation between days 3 and 5 could be due to either of two reasons or a combination of both. The first is that protein molecules, whose lifetime is extended in cells of old animals, have an increased chance of undergoing substantial structural modifications that render them available to degradative pathways which do not normally operate on 'native' forms. Several such pathways have been proposed by Knowles & Ballard (1976). An alternative proposal is that these modified proteins are more prone to degradation in autophagic vacuoles which involve lysosomal proteinases. Lysosomal cathepsin activity has been shown to increase with age in hepatocytes (Knook, 1980). These explanations should be considered speculative as long as the understanding of the nature of the protein-degradation pathways in cells remain obscure.

The results obtained with proteins in general, and particularly with the puromycinyl-peptides, suggest that there must be an age-related alteration in the function of the proteinases that are involved in protein degradation. This conclusion is based on the premise that the newly synthesized puromycinyl-

peptides are similar in young and old animals and should thus serve equally well as substrates for proteinases. These proteinases are presumably altered in old animals in the same manner as are many other enzymes (cf. Gershon, 1979). This phenomenon may explain, at least in part, the accumulation of altered enzyme molecules in old animals. The question of how this accumulation contributes to the general deterioration of essential cellular activities in old organisms is not entirely understood at present (for discussion see Gershon *et al.*, 1979).

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